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PRINCIPAL INVESTIGATOR: Zhou Wang, Ph.D.

CONTRACTING ORGANIZATION: Northwestern University
Evanston, Illinois 60208-1110

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Zhou Wang, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Northwestern University
Evanston, Illinois 60208-1110

E-Mail: wangz@northwestern.edu

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13. ABSTRACT (Maximum 200 Words)

Androgens are intimately associated with prostate cancer progression. We have previously identified more than 24 androgen-response genes. One of the genes encodes calreticulin, a highly conserved protein with demonstrated functions in intracellular Ca^{++} homeostasis, cell adhesion, chaperoning, and gene expression. Our studies have indicated that calreticulin overexpression is suppressive to anchorage-independent growth and metastasis of prostate cancer cells and calreticulin expression is down-regulated in human prostate tumor specimens. Thus, down-regulation of calreticulin in clinical prostate cancer specimens is likely to be an important step in prostate cancer progression. Our observations argue that part of androgen-induced gene expression program, such as calreticulin, is inactivated in the progression of prostate cancer, which represents a new concept in prostate cancer biology. Our results also provided strong basis for further exploring the mechanism by which calreticulin suppresses prostate tumor metastasis. In addition, we have generated several mutants for calreticulin, which will allow us to determine which of the three domains, N, P, or C, is responsible for the suppression of prostate tumor metastasis.

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Introduction:

Understanding the androgen action pathway in the prostate has clinical significance.

Androgen plays an important role in the development and progression of prostate cancer (Bosland, 1992; Carter and Coffey, 1990; Kozlowski and Grayhack, 1991; Lee et al., 1995). Understanding the androgen action pathway in the prostate will provide insights into the mechanisms by which androgen impacts the pathogenesis of prostate cancer, and may lead to more effective approaches for its prevention and treatment. The androgen action pathway here is defined as a cascade of molecular and cellular events triggered by androgen manipulation leading to cell proliferation, apoptosis, and/or differentiation.

Androgen controls homeostasis of the prostate.

As shown in Table 1, androgen stimulates proliferation and differentiation in a regressed prostate but not in a fully-grown prostate. On the other hand, androgen ablation induces massive apoptosis and rapid dedifferentiation in a fully-grown prostate, but has little or no effect on a regressed prostate. These observations suggest that in the regrowth process of a regressed prostate, androgen replacement stimulates and then nullifies proliferation, establishes apoptotic potential while inhibiting apoptosis, and induces and maintains differentiation. The molecular mechanisms by which androgen controls prostate regrowth remain largely unclear.

Table 1. The impact of androgen manipulation on the regressed prostate and the normal prostate.

Androgen	Regressed Prostate	Fully-Grown Prostate
+	Proliferation & Differentiation	No Significant Change
-	No Significant Change	Apoptosis & Dedifferentiation

+ represents androgen replacement and – represents androgen ablation or administration of anti-androgens. Differentiation is defined as the expression of prostate-specific markers. Dedifferentiation is defined as loss of the expression of prostate-specific markers.

Androgen action is mediated through androgen-response genes including calreticulin.

The dramatic influence of androgen on the prostate is mediated through androgen receptor (AR). AR is a ligand-dependent transcription factor that regulates the expression of androgen-response genes, either directly or indirectly (Mainwaring, 1977; Zhou et al., 1994). Thus, androgen-response genes should mediate AR downstream events leading to cellular and morphological changes in the prostate during androgen manipulation.

To study the androgen action pathway, we have searched for androgen-response genes on the basis of their induction during the initial regrowth of the regressed ventral prostate in 7-day castrated rats using a highly sensitive PCR-based cDNA subtraction method (Wang and Brown, 1991; Wang et al., 1997). Our search has identified 25 genes that are up-regulated by androgen and 4 genes that are down-regulated by androgen in the ventral prostate of a 7-day castrated rat.

One of the androgen-response genes encodes calreticulin. Our recent studies suggest that calreticulin has significant growth suppressive role in prostate cancer and its expression is down-regulated in prostate cancer cells. These observations argue that part of the androgen action pathway, which is growth suppressive, is down-regulated in prostate cancer pathogenesis.

Calreticulin is a multi-functional Ca^{++} binding protein.

Calreticulin is an evolutionarily conserved major Ca^{++} binding protein in endoplasmic reticulum ER (Krause and Michalak, 1997; Michalak et al., 1992; Sontheimer et al., 1995). Calreticulin has been implicated in the regulation of a variety of cellular functions including the regulation of intracellular Ca^{++} homeostasis (Bastianutto et al., 1995; Liu et al., 1994; Mery et al., 1996; Zhu and Wang, 1999), cell adhesion (Coppolino et al., 1995; Dedhar, 1994; Fadel et al., 1999; Opas et al., 1996), steroid-mediated gene regulation (Burns et al., 1994; Dedhar et al., 1994; Michalak et al., 1996), chaperone activity (Nauseef et al., 1995; Peterson et al., 1995; Vassilakos et al., 1998; Zapun et al., 1998), Zn^{++} binding, and rubella virus RNA binding. Calreticulin gene knockout mice are embryonic lethal because calreticulin is essential for cardiac development (Mesaeli et al., 1999).

Calreticulin consists of 400 a.a. residues after the N-terminal signal sequence is removed by posttranslational processing (Baksh and Michalak, 1996). It has a calculated MW of 46 kd and an apparent MW of 60 kd in SDS PAGE gel. There is a KDEL ER retention sequence at the C-terminal end of calreticulin. Calreticulin consists of at least 3 structural/functional domains (Fig. 1).

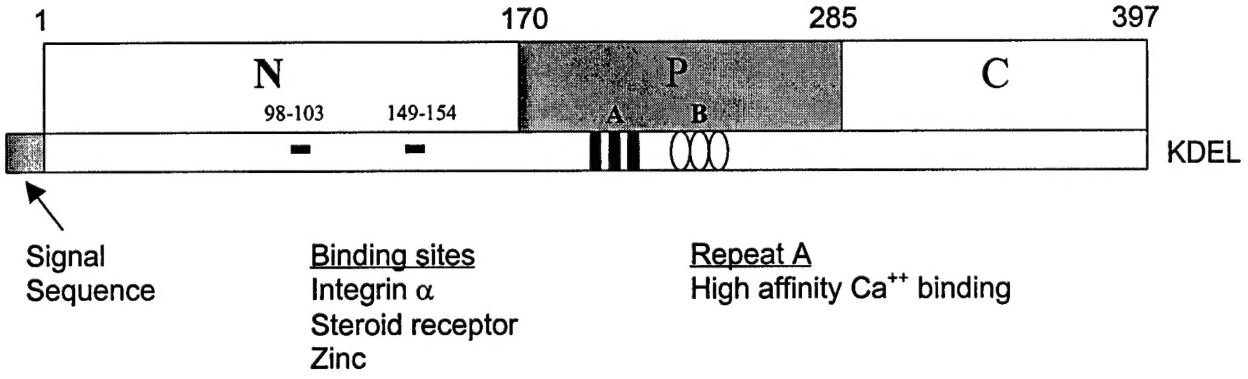


Fig. 1. The domain structure of calreticulin (Baksh and Michalak, 1996). The N-domain (aa 1-170) of calreticulin is the most conserved domain in evolution and does not bind to Ca^{++}. The N-domain forms a globular domain with 2 regions of short α-helices at residues 98-103 and 149-154, which are responsible for the binding to integrin α and the DNA binding domain of steroid receptors. The P-domain (aa 171-285) is proline-rich and contains two sets of repeats (Repeat A and Repeat B). Repeat A consists of three repeats of aa sequence PXXIXDPDAXKPEDWDE and is believed to be responsible for high affinity (Kd = 1.6 uM) and low capacity (1 Ca^{++}/protein) Ca^{++} binding to calreticulin. Repeat B consists of three aa sequence GXWXPPXIXNPXYX and is predicted to have a rigid turn structure separating the globular head of the protein from the acidic tail. The C-domain (aa 286-397) is highly acidic and negatively charged. This large stretch of negatively charged residues binds Ca^{++} with low affinity (Kd = 0.3-0.2 mM) and high capacity (~25 Ca^{++}/protein). These high capacity low affinity Ca^{++} binding sites have led to the hypothesis that calreticulin is involved in luminal Ca^{++} storage. The C-domain in calreticulin has the most divergent aa sequence among different species.

Calreticulin is abundantly expressed and regulated by androgen in prostate epithelial cells.

We became interested in calreticulin because it was identified in our search for androgen-response genes in the rat ventral prostate (Wang et al., 1997; Zhu et al., 1998). Androgen ablation by castration rapidly down-regulates calreticulin at both mRNA and protein levels for more than 10-fold. In contrast, androgen replacement rapidly restores the expression of calreticulin in the regrowth of the castrated prostate. Northern blot analysis of tissue-

specificity of calreticulin expression showed that calreticulin expression in the prostate is much more abundant than its expression in any other surveyed organs including liver, kidney, brain, heart, muscle, and seminal vesicles. In situ hybridization and immunohistochemistry studies demonstrated that calreticulin is an intracellular protein in the epithelial cells of the prostate. Calreticulin expression in human epithelial cells is also regulated by androgens, suggesting that calreticulin regulation by androgen is conserved in evolution (Zhu et al., 1998; Zhu and Wang, 1999).

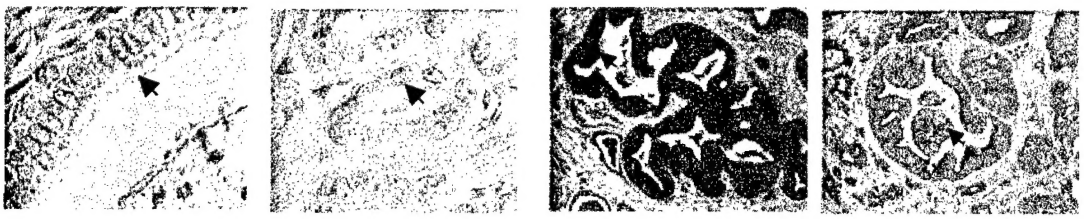
Body:

Task 1: Test the hypothesis that calreticulin down-regulation is more frequent in high Gleason grade prostate tumors (months 1-36).

a. Collect clinical specimens (months 1-36).
b. Calreticulin expression will be determined by IHC. The timing, magnitude, and frequency of calreticulin down-regulation in clinical prostate cancer specimens will be determined (months 1-36).
c. Statistical analysis will be applied to determine whether calreticulin down-regulation correlates with the Gleason grade (months 30-36).

Calreticulin expression is down-regulated in human prostate cancer specimens.

Expression of calreticulin in 21 hormone naïve clinical prostate specimens from radical prostatectomy was examined by immunohistochemistry (IHC) using an anti-calreticulin antibody (Zhu et al., 1998). These specimens contain benign regions, tumors, and/or high grade prostatic intraepithelial neoplasia (HGPIN). Calreticulin expression was down-regulated, to various extent, in 4 out of 11 HGPIN, 4 out of 10 Gleason 3 prostate tumors, and 2 out of 3 Gleason 4 prostate tumors. Examples of typical down-regulation are shown in Fig. 2. No calreticulin down-regulation was observed in benign prostatic epithelial cells in all of the specimens. These observations suggest that calreticulin down-regulation is more frequent in tumors with high Gleason score, which is associated with poor prognosis (Gleason and Mellinger, 1974). One clinically significant question is whether the cancer cells with loss of calreticulin expression will become highly metastatic and life-threatening.



A) Benign **B) Gleason 3** **C) Benign** **D) HGPIN**

Fig. 2. IHC studies of calreticulin expression in clinical prostate tumor specimens. One specimen containing both benign prostate (A) and Gleason 3 cancerous prostate (B) was stained with anti-Crt antibody and hemotoxylin as described previously (Zhu et al., 1998). Another specimen containing both benign prostate (C) and HGPIN (D) was stained with anti-Crt antibody but without hemotoxylin. Secondary antibody alone did not stain the section (Results not shown). The benign and cancerous epithelial cells are marked with arrows.

Table 1. Calreticulin immunostaining intensity in 21 human prostate tumor specimens.

Intensity	Benign (n=21)	HGPIN(n=11)	Gleason 3 (n=10)	Gleason 4 (n=3)
+	0	0	10%	33.3%
++	0	36.4%	30%	33.3%
+++	100	63.6%	60%	33.3%

The intensity of the staining was determined using specimens stained with anti-calreticulin without hemotoxylin. +++ represents normal level staining; ++ represents moderate down-regulation; + represents barely detectable expression of calreticulin.

Recently, we have extended our studies using prostate cancer tissue array. The arrays contained 124 tissue cores derived from 42 patients. The staining intensity was graded from 0 to 4 in a blinded fashion as described by Grizzle et al (Grizzle et al., Immunohistochemical Evaluation of Biomarkers in Prostatic and Colorectal Neoplasia). The down-regulation of calreticulin was reproduced and the extent of down-regulation correlates with Gleason grade (Fig. 3).

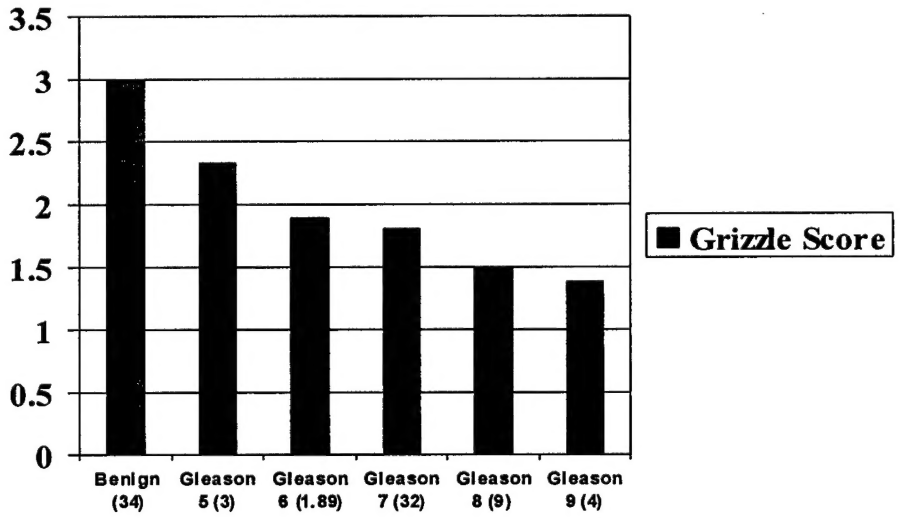


Fig. 3. Calreticulin staining intensity of benign or cancerous human prostate tissue specimens in tissue microarray. The Gleason scores of tumor specimens were indicated.

Task 2: Determine the functional domains and motifs of calreticulin in inhibiting anchorage-independent growth (months 1-36).

a. Deletion mutants will be generated to map domain(s) essential for inhibiting anchorage-independent growth of prostate cancer cells (months 1-24).
b. Substitution mutants will be generated to map essential motifs (months 1-24).
c. The impact of calreticulin mutants on the growth of PC3 prostate cancer cells in soft agar will be tested to identify essential amino acid sequences in calreticulin (months 25-36).

Construction of mutant calreticulin expression vectors.

To prepare for mechanistic studies, we have constructed a series of mutant calreticulin expression vectors. As illustrated in Fig. 1, calreticulin consists of 3 structural domains, N, P, and C. Dr. Marek Michalak has kindly provided us with HA tagged wild-type rabbit calreticulin (Rb Crt-HA) and 3 HA-tagged mutants (Rb N-HA, Rb N+P-HA, and Rb P-HA). We have made additional HA-tagged mutant calreticulin expression vectors. In addition, we have also generated GFP-tagged mutant calreticulin expression vectors (Fig. 4), which will allow us to conveniently determine the intracellular localization of various calreticulin mutants.

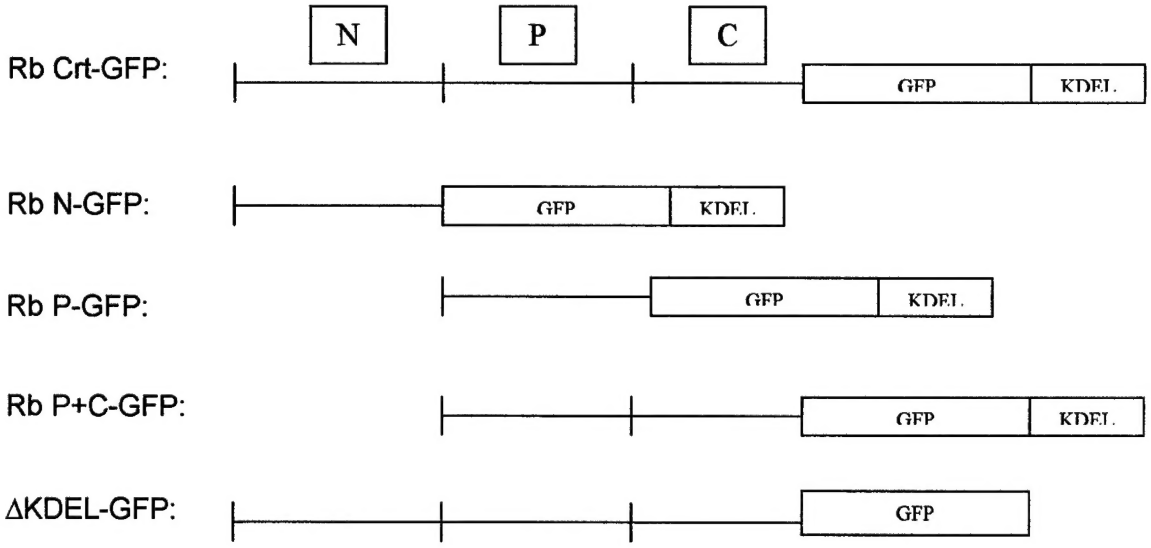


Fig. 4. Green fluorescent protein (GFP)-tagged wild-type calreticulin and calreticulin mutants. The rabbit calreticulin was used in the mutant construction. N, P, and C stand for N-domain, P-domain, and C-domain respectively (See Fig. 1 for details). All of the constructs were verified by sequencing analysis. All of the constructs have the signal peptide sequence at their N-terminus.

Crt-GFP colocalizes with an ER marker (PDI)

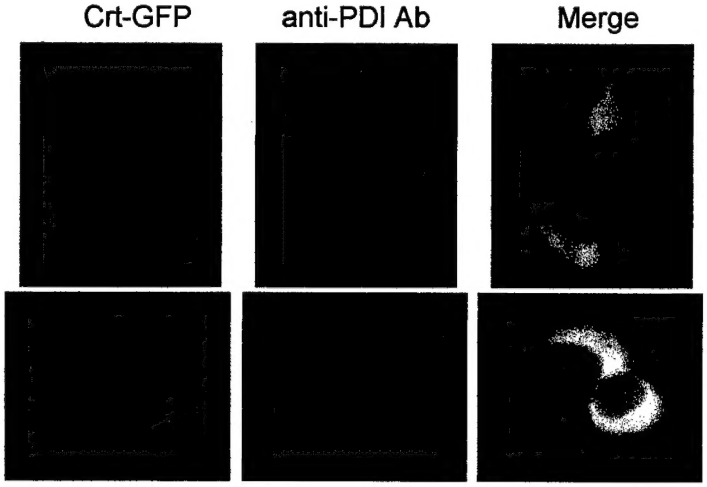


Figure 5. Co-localization of Crt-GFP with protein disulfide isomerase (PDI), an ER-resident protein. Crt-GFP/pcDNA3.1 was transiently-transfected into PC3 cells using Fugene 6.0. Cells were plated onto coverslips and fixed in 4% paraformaldehyde. Immunofluorescence was performed using an α-PDI antibody (Stressgen).

In the course of our investigation, we realized one important issue regarding calreticulin mutants - whether mutations will affect intracellular localization of calreticulin. As expected, wild-type calreticulin is colocalized with PDI, an ER marker (Fig. 5). If a mutation altered the intracellular localization of calreticulin, it is likely to have a dramatic impact on calreticulin function. It will be important to for us to conduct functional analysis on calreticulin mutants that are still localized in endoplasmic reticulum. Thus, we have carried out extensive analysis on the intracellular localization of various GFP-tagged calreticulin mutants. The surprising finding is that the KDEL ER-retention signal is not necessary for proper calreticulin localization (Fig. 6). The P and C domain contains signals for ER localization (Fig. 6), which was not expected. These findings will allow us to focus on mutants containing P and/or C domain.

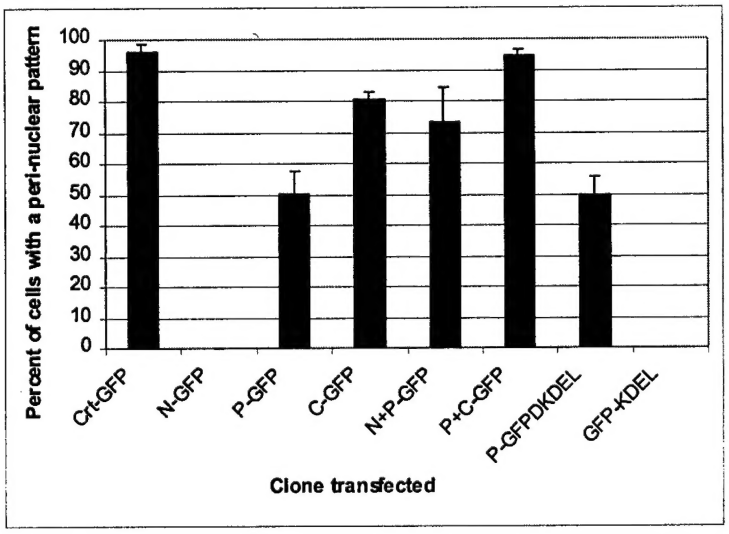
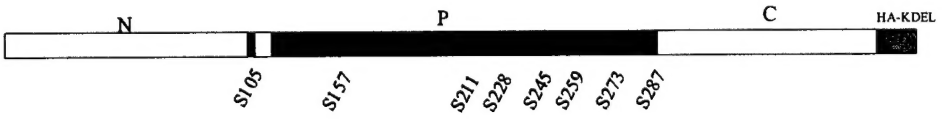


Figure 6. Quantification of localization of Crt-GFP and domain mutants fused to GFP. Crt and its domain mutants fused to GFP were transiently transfected into PC3 cells using Fugene 6.0. After 24 hrs, the number of cells showing peri-nuclear compartment localization was counted (minimum of 100 cells per sample).

In addition, we have generated 8 substitution mutants in the conserved domains (Fig. 7). These substitution mutants will allow us to define the functional significance of the conserved domains.

A.



B.

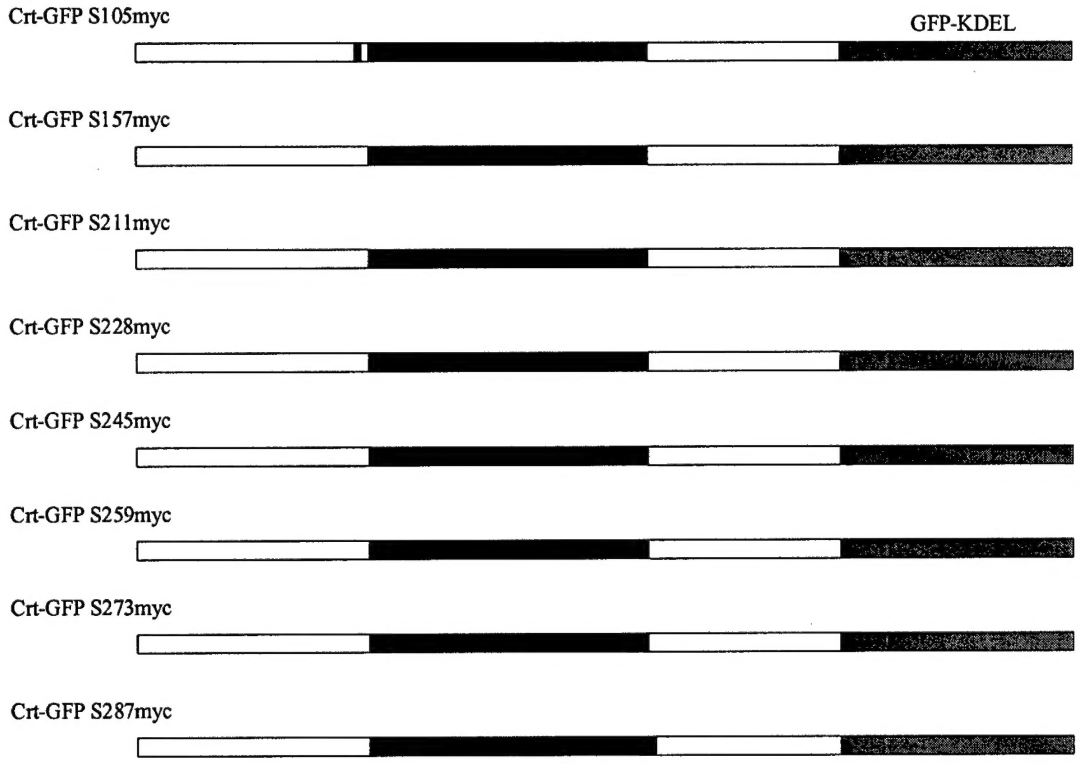


Fig. 7. Substitution mutants in 8 conserved calreticulin domains. A. Positions of 8 conserved amino acid stretches (10-11 amino acid residues in length). These positions in calreticulin were replaced with the myc epitope tag individually. **B.** GFP-tagged substitution mutants. Various substitution mutants were fused to GFP using a PCR-based approach. All the mutants were verified by sequencing.

Endogenous calreticulin is expressed in all of the prostate cancer cell lines, including PC3, which may influence the functional analysis of exogenous wild-type and/or mutant calreticulin. To facilitate our functional studies, we have generated siRNA expression vector for human calreticulin. Stable PC3 sublines with down-regulated endogenous calreticulin were generated (Fig. 8), which will help us carry out overexpression studies of various calreticulin mutants.

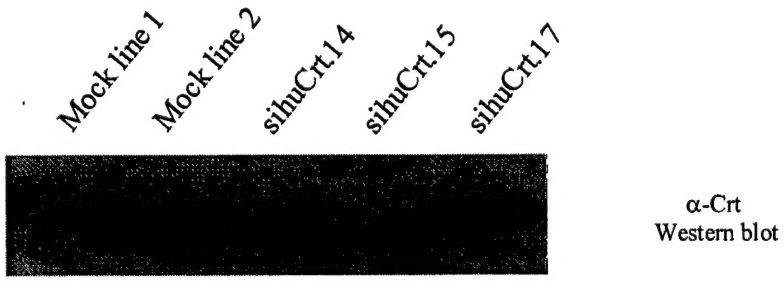


Fig. 8. Western blot of down-regulated Crt PC3 lines. Equal amounts of protein were loaded on 10% SDS-PAGE, transferred to nitrocellulose, and probed using an α-Crt monoclonal antibody (Stressgen).

Task 3: Study the role of calreticulin in prostate tumor growth and metastasis *in vivo* in tumor xenografts (months 1-36).

a. The effect of wild-type calreticulin on the growth and metastasis of subcutaneous and orthotopic xenograft prostate tumors will be studied in 32 nude mice (months 1-12).
b. The effect of calreticulin deletion mutants on the growth and metastasis of subcutaneous and orthotopic xenograft prostate tumors will be studied in 56 nude mice (months 13-24).
c. The effect of calreticulin substitution mutants on the growth and metastasis of subcutaneous and orthotopic xenograft prostate tumors will be studied in 56 nude mice (months 25-36).

In previous progress report, we have showed that calreticulin markedly inhibits anchorage-independent growth of prostate cancer cells. In addition, we showed that calreticulin expression inhibits metastasis of rat Dunning prostate cancer cells in vivo.

In the past year, we were unable to accomplish proposed Aim 3b. This is mainly due to the fact that we must resolve the intracellular localization of various calreticulin mutants prior to their further functional analysis. Now we have showed that P and C domain are essential for proper localization of calreticulin, which itself is an important discovery. We plan to focus our functional analysis on calreticulin mutants with proper intracellular localization.

Key Research Accomplishments:

1. Calreticulin expression is down-regulated in human prostate cancer specimens.

Our hypothesis states that calreticulin is suppressive to prostate cancer malignancy. If calreticulin suppression of prostate cancer progression is important *in vivo*, the expression of calreticulin should be down-regulated in clinical prostate cancer specimens. Our studies provided evidence for the down-regulation of calreticulin *in vivo* in clinical specimens, which is critical to this project.

2. Construction of calreticulin substitution mutant expression vectors.

Substitution mutagenesis is a powerful approach for elucidating the motif(s) that is critical for protein function(s). To identify motif(s) in calreticulin responsible for the suppression of anchorage-independent growth and/or metastasis of prostate cancer cells, we have generated 8 calreticulin substitution mutants either tagged with GFP. These mutants will provide insights into the mechanism by which calreticulin suppresses metastasis.

3. P and C domains in calreticulin are critical for proper intracellular localization of calreticulin.

To facilitate our functional studies proposed in specific aim 3, it is important to assess the impact of various mutations on calreticulin intracellular localization. Our data showed that the P and/or C domain containing calreticulin mutants are capable of localizing to endoplasmic reticulum. We plan to focus on calreticulin mutants with endoplasmic reticulum localization in our future studies.

4. Generation of stable PC3 sublines with down-regulated calreticulin expression.

Endogenous careticulin is expressed in all the assayed cell lines, which could interfere, to various extent, functional studies of calreticulin mutants. The availability of PC3 sublines with calreticulin expression down-regulated by siRNA should facilitate our analysis of various calreticulin mutants by transfection. The siRNA is specific for human calreticulin and calreticulin mutants are derived rabbit or rat calreticulin, which are not targeted by the siRNA.

Reportable Outcomes:

1. A manuscript entitled "Calreticulin is a potential metastasis suppressor in prostate cancer" is in preparation.
2. A manuscript entitled "Identification and characterization of domains responsible for endoplasmic reticulum localization of calreticulin" is in preparation.

Conclusions:

Our studies have indicated that calreticulin overexpression is suppressive to anchorage-independent growth and metastasis of prostate cancer cells and calreticulin expression is down-regulated in human prostate tumor specimens. In the last funding period, we have generated 8 substitution mutants for calreticulin, which will allow us to determine which of the conserved motifs is responsible for the suppression of prostate tumor metastasis. In addition, we have discovered that the P and C domain, rather than the KDEL signal, is critical for calreticulin to localize to endoplasmic reticulum. Our finding will allow us to focus our functional analysis on calreticulin mutants with proper intracellular localization.

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